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(54) Title: METHODS AND COMPOSITIONS FOR PREVENTING IMMUNE REJECTION OF SOLID ORGAN GRAFTS

(57) Abstract

The present invention provides methods for tolerizing a recipient for solid organ transplantation, comprising the steps of (a) harvesting cells from an anticipated donor of a solid organ, the cells including hematopoietic stem cells, (b) enriching hematopoietic stem cells from the harvested cells, and (c) infusing the enriched stem cells into a recipient, such that a state of tolerance to a transplanted solid organ from the donor is induced in the recipient.

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Description

METHODS AND COMPOSITIONS FOR PREVENTING IMMUNE REJECTION OF SOLID ORGAN GRAFTS

Technical Field

The present invention relates generally to solid organ transplantation and, more specifically, to methods and compositions suitable for inducing donor antigen-specific immunological tolerance in recipients of solid organ grafts.

Background Of The Invention

The science of transplantation of human organs has advanced to the point where more than 10,000 solid organ transplants, including heart, kidney, liver, and lung transplants, are performed in the U.S. annually. The vast majority of these are cadaveric transplants, although some transplants, such as kidney transplants, are from living, usually related, donors. Solid organ transplantation can be a highly cost-effective method for treating various diseases. For example, for end-stage renal disease kidney transplantation may be performed at approximately one-third of the cost of dialysis over the life of the patient (*New Engl. J. Med. 17*:1243, 1991).

It is axiomatic that successful organ transplantation requires the long-term administration of immunosuppressive drugs, such as cyclosporine A, cyclophosphamide, azathioprine, corticosteroids, and anti-lymphocyte serum (ALS), among others. The advent of cyclosporine, in particular, has dramatically expanded the number of transplants (Stratta et al., *Transplantation 45*:40, 1988). Nonetheless, it is widely agreed that graft survival, especially of xenografts, remains suboptimal. For example, it is estimated that 20% of allogeneic kidney grafts fail within one year of transplant, and that 80% fail within ten years. Even worse are the statistics for other organ grafts.

Another acknowledged need in the art is the development of alternatives to chronic immunosuppressive therapy of transplant recipients. This is needed both to improve graft acceptance (Barber, *Transplant. Rev. 4*:68, 1990; Thomas et al., *Transplant. Proc. 23*:11, 1991) and to reduce the morbidity and mortality associated with nonspecific immunosuppression in patients with long-term organ engraftment (Mahony et al., *Med. J. Australia 2*:426, 1982; Weir et al., *Kidney Int. 28*:839, 1985; Kelly et al., *Clin. Transplant. 1*:271, 1987; Rao and Anderson, *Transplantation 45*:45, 1988).

Studies performed almost fifty years ago demonstrated conclusively that the rejection of organ allografts is an immunological phenomenon (Medawar, *J. Anat. 78*:176, 1944). At the same time, it was shown that dizygotic cattle which share a placenta (so-called freemartins) are rendered tolerant to reciprocal skin grafts by virtue of their exposure to each other's erythrocytes *in utero* (Owen, *Science 102*:460, 1945; Anderson et al., *Heredity 5*:379, 1951). This observation was further supported by studies showing that tolerance to allografts could be induced in neonatal animals by the deliberate infusion of donor lymphoid cells (Billingham et al., *Nature 172*:603, 1953).

Such data provided the impetus for attempts to induce donor antigen-specific tolerance to solid organ allografts, for example, by random donor blood transfusions (reviewed in Opelz, *Transplant. Proc. 17*:1015, 1985), donor-specific blood transfusions (Salvatierra et al., *Transplantation 34*: 326, 1981; van Twuyver et al., *New Engl. J. Med. 17*:1210, 1991), and donor bone marrow transfusions (Monaco et al., *Heart Transplant. 1*:257, 1982; Monaco et al., *Transplant. Proc. 17*:1312, 1985). Collectively, these and related studies suggested that the establishment of mixed chimerism (a state in which there is a stable mixture of host and donor hematopoietic elements) in the transplant host leads to persistent tolerance to other donor tissues. The challenge then, as now, is to produce a durable chimeric state without eliciting graft versus host disease (GVHD) or precipitating acute organ rejection.

Currently, the preferred method of achieving a mixed chimeric state is to induce myelosuppression in the host by sublethal or total lymphoid irradiation, or by treatment with anti-lymphocyte serum (ALS), followed by transfusion of unfractionated donor bone marrow. This approach has been evaluated in numerous animal models (see, for example, Hartner et al., *Transplantation 52*:784, 1991; Wren et al., *J. Pediat. Surg. 26*:439, 1991; Ildstad et al., *J. Surg. Res. 51*:372, 1991; Ildstad et al., *Transplantation 51*:1262, 1991; van den Brink et al., *Transplant. Proc. 23*:1807, 1991).

This approach has also been evaluated clinically by Barber and colleagues (*Transplant. Rev. 4*:68, 1990; *Transplantation 51*:70, 1991). Briefly, Barber induced immunosuppression in renal transplant recipients with anti-lymphocyte globulin (ALG), beginning the day after transplantation and continuing for 10-14 days thereafter. In addition, all patients were maintained on an immunosuppressive regimen of cyclosporine, azathioprine, and prednisone post-transplant. The results of these studies indicate that renal allograft survival in patients receiving donor bone marrow is significantly improved relative to historical controls and relative to patients receiving the contralateral kidney without concomitant bone marrow transfusion. However, the number of rejection episodes was not significantly different between the experimental and control groups.

Several groups have attempted to define the cell type in bone marrow responsible for inducing tolerance in the host. In mice, this cell appears to be a small mononuclear cell which is Ia-, Thy-1-, and FcR+ (Gozzo et al., *J. Immunol. 129*:1584, 1982; De Fazio et al., *J. Immunol. 135*:3035, 1985; De Fazio et al., *Transplant Proc. 19*:547, 1987). In the rhesus monkey, the relevant cell type appears to be DR-, CD3-, CD2+, and CD16+ (Thomas et al., *Transplant. Proc. 23*:11, 1991). Starzle et al. (*Immunol. Today 14*:326, 1993) have postulated that dendritic cells are prime candidates in tolerance induction. To date, there are few data, much less consensus as to the cell type(s) in human bone marrow which is (are) critical for tolerance induction.

There is therefore a need in the art for a method of inducing tolerance in recipients of solid organ grafts which eliminates the need for chronic non-specific immunosuppression of the host and improves graft survival. Ideally, such a tolerization method will not precipitate acute organ rejection and will also avoid the risk of GVHD. The present invention provides such methods, and additionally, provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides methods and compositions for preventing immune rejection of solid-organ grafts by tolerizing a recipient for solid-organ transplantation. Within one aspect of the present invention, methods of tolerizing a recipient for solid-organ transplantation are provided comprising the steps of (a) harvesting cells from an anticipated donor of a solid-organ, the cells including hematopoietic stem cells, (b) enriching hematopoietic stem cells from the harvested cells, and (c) infusing the enriched stem cells into a recipient, such that a state of tolerance to a transplanted solid-organ from the donor is induced in the recipient.

Within another aspect of the present invention, methods of tolerizing a recipient for solid-organ transplantation are provided comprising the steps of (a) harvesting cells from an anticipated donor of a solid-organ, the cells including hematopoietic stem cells, (b) enriching hematopoietic stem cells from the harvested cells, (c) conditioning a recipient for solid-organ transplantation, and (d) infusing the enriched stem cells into the conditioned recipient, such that a state of tolerance to a transplanted solid-organ from the donor is induced in the recipient.

Within other aspects of the present invention, methods are provided for transplanting solid organs comprising the steps of (a) harvesting cells from an anticipated donor of a solid-organ, the cells including hematopoietic stem cells, (b) enriching hematopoietic stem cells from the harvested cells, (c) infusing the enriched stem cells into an anticipated solid-organ graft recipient, such that a state of tolerance to

a solid-organ from the donor is induced in the recipient, and (d) transplanting an organ from the donor into the recipient.

Within yet another aspect of the present invention, methods are provided for transplanting solid organs, comprising the steps of (a) harvesting cells from an anticipated donor of a solid-organ, the cells including hematopoietic stem cells, (b) enriching hematopoietic stem cells from the harvested cells, (c) transplanting an organ from the donor into the recipient, and (d) infusing the enriched stem cells into the recipient, such that a state of tolerance to a solid-organ from the donor is induced in the recipient.

Within other aspects of the present invention, methods are provided for transplanting solid organs comprising the steps of (a) harvesting cells from an anticipated donor of a solid-organ, the cells including hematopoietic stem cells, (b) enriching hematopoietic stem cells from the harvested cells, (c) conditioning a recipient for solid-organ transplantation, and (d) infusing the enriched stem cells into the conditioned recipient, such that a state of tolerance to a solid-organ from the donor is induced in the recipient, and (e) transplanting an organ from the donor into the recipient.

Within yet another aspect of the present invention, methods are provided for transplanting solid organs, comprising the steps of (a) harvesting cells from an anticipated donor of a solid-organ, the cells including hematopoietic stem cells, (b) enriching hematopoietic stem cells from the harvested cells, (c) conditioning a recipient for solid-organ transplantation, (d) transplanting an organ from the donor into the conditioned recipient, and (e) infusing the enriched stem cells into the conditioned recipient, such that a state of tolerance to a solid-organ from the donor is induced in the recipient.

Within one embodiment of the invention, subsequent to the step of enriching the stem cells and prior to the step of infusing the enriched stem cells into a conditioned recipient, the invention further comprises the steps of (a) freezing the enriched stem cells, and (b) thawing the frozen stem cells.

Within other embodiments of the invention, the recipient may be conditioned by administration of an immunosuppressive agent selected from the group consisting of an anti-lymphocyte serum, anti-thymocyte globulin, anti-lymphocyte globulin, cyclosporine A, FK506, azathioprine, and cyclophosphamide. Alternatively (or in combination with the above) the recipient may be conditioned by administration of a corticosteroid or CD3 monoclonal antibody, or by irradiation.

Within yet other embodiments of the invention, the solid-organ is selected from the group consisting of kidney, lung, liver, heart, pancreas and skin and multi-visceral, and the harvested cells are selected from the group consisting of bone

marrow, mobilized peripheral blood, fetal liver, and cord blood. Within other embodiments, the hematopoietic stem cells may be enriched from the harvested cells by immunoselection. Within yet other embodiments, the invention further comprises, subsequent to the step of enriching the stem cells, expanding the number of stem cells *in vitro*.

Within preferred embodiments of the invention, the hematopoietic stem cells are $CD34^+$ cells. As will be readiiy understood by one of ordinary skill in the art given the disclosure provided herein, homologues of $CD34^+$ cells exist in animals other than humans, and thus are preferably utilized within other embodiments of the invention (*e.g.*, for xenograft transplantations).

These and other aspects of the present invention will become evident upon reference to the following detailed description. In addition, various references are set forth below which describe in more detail certain procedures or compositions. These references are incorporated herein by reference in their entirety.

Detailed Description of the Invention

As noted above, the present invention provides methods and compositions (also referred to as "medicaments") for preventing immune rejection of solid-organ grafts by tolerizing a recipient for solid-organ transplantation. Among the advantages which this invention provides are a reduction in the duration of, or elimination of, chronic immunosuppressive therapy, and in addition, improved graft survival.

Within one aspect of the present invention, methods are provided for tolerizing a recipient for solid-organ transplantation comprising the steps of (a) harvesting cells from an anticipated donor of a solid-organ, the cells including hematopoietic stem cells, (b) enriching hematopoietic stem cells from the harvested cells, and (c) infusing the enriched stem cells into a recipient, such that a state of tolerance to a transplanted solid-organ from the donor is induced in the recipient. Within the context of this disclosure it should be understood that a state of tolerance to a transplanted solid organ-donor has been induced if tolerance to donor antigens has been induced in the recipient. This includes the situation in which two-way tolerance (donor tolerant of recipient and recipient tolerant of donor) is induced. Representative assays for determining induction of tolerance to donor antigens are described in more detail below.

As noted above, a population of cells containing hematopoietic stem cells are harvested from an anticipated donor of a solid-organ. Briefly, within the context of the present invention hematopoietic stem cells are defined as those cells which are

capable of both replacing themselves and of giving rise to all of the cells of the hematopoietic lineages, including myeloid, erythroid, lymphoid and megakaryocytic. They may also be defined by their antigenic makeup. For example, human hematopoietic stem cells are known to express the CD34 antigen, among other antigens, at the cell surface and to lack lineage-specific antigens, such as B lymphocyte markers, T lymphocyte markers, etc..

The CD34 antigen is present on human hematopoietic stem cells and progenitor cells, but is absent from mature hematopoietic cells (reviewed in Knapp, W et al., eds., *Leucocyte Typing IV*, Oxford: Oxford UP, pp. 816-830, 1989; and Sutherland and Keating, *J. Hematotherapy 1*:115-129, 1992). That this marker is not completely specific for stem cells (as opposed to progenitor cells) is believed to be inconsequential in the method of the instant invention, provided that the marker chosen does not identify mature hematopoietic cells.

In addition to CD34, other antigens which may be useful for identifying and selecting hematopoietic stem cells include various growth factor receptors, such as the receptor for stem cell factor (SCF).

Hematopoietic stem cells may be harvested from any of a variety of tissues, including for example, fetal liver and spleen, umbilical cord blood, bone marrow of living or cadaveric organ donors, and peripheral blood of living donors. Bone marrow is a particularly rich source of stem cells (typically 1-2% of marrow mononuclear cells are $CD34^+$), and is typically obtained by aspiration (under general anesthesia, if from a living donor) from the iliac crest, but may be obtained from other sites (such as the sternum, ribs, and vertebral bodies) if necessary. Generally, marrow is obtained at the same time as the organ to be transplanted.

Hematopoietic stem cells may also be obtained from peripheral blood. In particular, although peripheral blood contains fewer stem cells (typically < 1% of peripheral blood mononuclear cells are $CD34^+$) than bone marrow, it is generally easier to obtain from a living donor. Within one embodiment of the invention, the number of stem cells circulating in peripheral blood may be increased in the donor by prior exposure of the donor to certain growth factors, such as G-CSF, GM-CSF, IL-1 or SCF; to certain drugs, such as 5-fluorouracil; and/or to certain antibodies, such as anti-VLA4. Peripheral blood collected from donors who have been pre-treated in this manner to increase the number of circulating $CD34^+$ cells are said to have been "mobilized." Depending upon the volume which is desired, blood may be collected by venipuncture or by one or more aphereses. Typically, an anticoagulant is added to the blood, such as acid-citrate-dextrose (ACD), ethylenediamine tetraacetic acid (EDTA), heparin, or citrate-phosphate-dextrose-adenine (CPDA). Peripheral blood may be

obtained at the same time as the organ to be transplanted or it may be obtained at a later time, for example, sufficiently in advance of infusion to allow for processing but not so far in advance as to necessitate cryopreservation.

Enrichment of Hematopoietic Stem Cells

In order to enrich hematopoietic stem cells from the harvested cells, it is generally preferred to first prepare a buffy coat or mononuclear cell (MNC) fraction from the bone marrow or peripheral blood specimen in order to reduce the number of contaminating red cells in the specimen. Methods for the preparation of buffy coats and mononuclear cell fractions are well-known in the art and are described, for example, in Kumar and Lykke, *Pathology 16*:53, 1984; Boyum, *Scand. J. Clin. Lab. Invest. 21*:77, 1968; and Mishell and Shiigi, eds., *Selected Methods in Cellular Immunology*, San Francisco: WH Freeman, p. 186-208, 1980.

A peripheral blood or bone marrow specimen, or buffy coat or mononuclear cell fraction thereof, may also be enriched for hematopoietic stem cells by a method such as counterflow centrifugal elutriation or immunoselection. Briefly, in counterflow centrifugal elutriation, cells are fractionated according to size, density, or a combination of both, using a rotor especially designed for this purpose (such as the JE-10X from Beckman Instruments, Palo Alto, CA). Typically, four different fractions are collected, of which the fraction collected when the rotor is stopped but medium is still flowing is generally regarded as the progenitor (stem cell) -rich fraction (Noga et al., in *Bone Marrow Purging and Processing*, NY: Alan R. Liss, p. 345, 1990).

For use within the present invention however, immunoselection is generally preferred over centrifugal elutriation because both the yield and purity of stem cells is superior, and because sterility can be more easily maintained. Immunoselection may be accomplished by negative immunoselection, positive immunoselection, or a combination of the two. Briefly, "positive" selection refers to the capture of cells by some means, usually immunological, on the basis of their expression of a specific characteristic or set of characteristics (usually an antigen(s) or receptor(s) expressed at the cell surface). "Negative" selection refers to the exclusion or depletion of cells by some means, usually immunological, on the basis of their lack of expression of a specific characteristic or set of characteristics (again, usually a surface antigen(s) or receptor(s)).

In one embodiment of the invention, hematopoietic stem cells are positively immunoselected on the basis of their expression of the CD34 marker utilizing a polyclonal or monoclonal anti-CD34 antibody, or fragment thereof. A variety of anti-CD34 antibodies have been described in the literature, many of which are commercially available. These include 12.8 (Andrews et al., *Blood 67*:842, 1986), My10 (Civin et al.,

J. Immunol. *133*:157, 1984; commercially available from Becton Dickinson under the designation HPCA-2), QBEND-10 (Fina et al., *Blood* *75*:2417, 1990; commercially available from Quantum Biosystems, Cambridge, England), B1.3C5 (Katz et al., *Leuk. Res.* *9*:191, 1985); ICH3 (Watt et al., *Leukaemia* *1*:417, 1987); and TUK3 (Unchanske-Ziegler et al., *Tissue Antigens* *33*:230, 1989).

Alternatively, hematopoietic stem cells may be negatively selected on the basis of their lack of expression of lineage-defining antigens. For example, a cocktail of monoclonal or polyclonal antibodies, or fragments thereof, which includes antibodies to myeloid, erythrocytic, lymphoid, and megakaryocytic lineages, can be used to bind $CD34^-$ cells. Among lineage-defining antigens antibodies to which may be useful for negative selection of CD34 cells are CD15 and CD33 (myeloid markers); CD19, CD20, CD4, CD8, CD10, and CD45 (lymphoid markers); glycophorin (erythroid marker), etc. These and other markers, and antibodies thereto are described, for instance, in Knapp et al. (op. cit.).

Immunoselection of hematopoietic stem cells may be accomplished by any of a variety of means known to those skilled in the art, including immunoaffinity chromatography, fluorescence activated cell sorting, panning (Wysocki and Sato, *Proc. Natl. Acad. Sci. (USA)* *75*:2844, 1978), magnetic activated cell sorting (Miltenyi et al., *Cytometry* *11*:231, 1990), and cytolysis. Generally, immunoselection of a heterogeneous population of cells, such as in a bone marrow aspirate or a peripheral blood specimen or apheresis product, to yield target and non-target fractions is rarely complete. For the purposes of the present invention, a given target cell population (*e.g.*, $CD34^+$ cells) is considered to have been enriched if the ratio of target to non-target cells in the enriched fraction is at least 10X the ratio of target to non-target cells in the starting population. Most preferably, the ratio of target to non-target cells in the enriched fraction will be 30-100X the ratio of target to non-target cells in the starting population.

Particularly preferred methods of immunoselection are described in the following U.S. patents, all of which are herein incorporated by reference: U.S. 5,215,927, entitled "Method of Immunoselection of Cells using Avidin and Biotin," issued June 1, 1993 to Berenson et al., and U.S. 5,225,353, entitled "Method of Immunoselection of Cells Using Avidin and Biotin," issued July 6, 1993 to Berenson et al.. Briefly, a heterogeneous population of cells is labeled, either directly or indirectly, with a biotinylated antibody specific for a targeted subpopulation of the cells. The resultant labeled cells are then flowed, without static incubation, through a column containing immobilized avidin, such that the labeled cells are substantially bound to the immobilized avidin. The labeled cells can be recovered from the column, if desired, in a

subsequent step, by agitating the solid phase such that the cells are released in substantially viable condition.

The above-described methods are preferred over other methods of immunoselection for several reasons. Briefly, because the biotin-labeled cells are not incubated under static (substantially non-flowing) conditions with the solid phase (immobilized avidin), non-specific binding (of unlabeled cells to the solid phase) is dramatically reduced. Further, the nature of the interaction between avidin and biotin under the conditions described in the above-cited patents is such that a substantial fraction of the target cell population is captured. This capture efficiency, or yield, is particularly important when one is attempting to select a rare population of cells, such as hematopoietic stem cells, from among more abundant cell types present in a heterogeneous mixture, such as a blood or bone marrow specimen. Yet a further advantage resides in the ability to recover substantially all of the cells which are bound to the solid phase in a condition suitable for transplantation. Cells are said to be suitable for transplantation if they are substantially viable and substantially biologically active. In addition, it is preferable that the cells be free of surface antibody, especially mouse antibody, which may induce a human anti-mouse antibody (HAMA) response in transplant host. These conditions are generally provided by the methods described in the above-referenced patents or in PCT/US91/02785, entitled "Methods for Removing Ligands from a Particle Surface," published on October 31, 1991, which is herein incorporated by reference.

Immunoselection Devices Useful for the Enrichment of Hematopoietic Stem Cells

Immunoselection may be performed utilizing devices such as those described in co-pending patent applications U.S.S.N. 08/005,891, entitled "Improved Apparatus and Method for Cell Separation," and U.S.S.N. 07/599,796, entitled "An Apparatus and Method for Separating Particles Using a Pliable Vessel," both of which are herein incorporated by reference. Briefly, the '891 application describes a cell separator, including a column assembly for separating target cells from a sample fluid, the column assembly including a column, a sample fluid supply bag and a fluid collection bag wherein the column is provided for receiving the sample fluid from the sample fluid supply bag and for separating the target cells from the sample fluid and retaining the target cells, and wherein the fluid collection bag is provided for receiving the target cells after being released from the column, said cell separator comprising an agitation means for agitating the contents of the column to assist in releasing the sample cells retained in the column, the agitation means being responsive to a drive signal for varying the amount of agitation of the contents of the column to vary the rate at which the sample

cells are released, column sensor means for providing a column signal indicative of the optical density of fluid flowing out of the column and into the fluid collection bag, a column valve means responsive to a column valve control signal for selectively enabling the fluid coming out of the column to flow into the fluid collection bag, and a data processor means for controlling the operation of the cell separator, the data processor means being responsive to the column signal for providing the drive signal and the column valve control signal to prevent inadequate concentrations of the target cells from being collected. One embodiment of this invention is the CEPRATE SC cell separation system which is manufactured by CellPro (Bothell, WA).

The '796 application describes a vessel having an inlet through which a mixture of target and non-target cells may be introduced and an outlet through which the fluid may exit, at least a portion of which is pliable, and a bed of binding material disposed within the vessel, the binding material attracting the target cells such that they become bound thereto and being porous enough to allow the non-target cells to pass therethrough. Deformation of the pliable portion of the vessel causes relative movement in the binding material, thereby creating the necessary degree of agitation to cause the target cells to become dislodged from the binding material. One embodiment of this invention is the CEPRATE LC laboratory column, which is commercially available from CellPro.

Expansion and Preservation of Enriched Hematopoietic Stem Cells

In many instances, it may be desirable to expand (increase in number) the enriched hematopoietic stem cells *in vitro* in order to ensure a sufficient number of cells for tolerance induction. Within one embodiment of the invention, this may be accomplished by inoculating the enriched cells into a suitable vessel, containing a nutritive medium supplemented with a source of growth factors and, optionally, human or other animal plasma or serum, and incubating the resultant culture in a humidified atmosphere containing approximately 5% CO_2 for a period of time sufficient to yield the desired increase in cell number.

A particularly preferred method of expanding hematopoietic stem cells is described in co-pending patent application U.S.S.N. 08/008,716, entitled "Methods and Device for Culturing Human Hematopoietic Cells and their Precursors in Suspension," which is herein incorporated by reference. Briefly, enriched hematopoietic stem cells are inoculated into a culture vessel containing a culture medium comprising a nutritive medium and a source of growth factors and cultured under agitative conditions sufficient to maintain the cells substantially in suspension and for a time sufficient to increase the number of said cells.

In many instances, it may also be desirable to preserve enriched hematopoietic stem cells for a period of time sufficient to permit conditioning of a solid organ graft recipient prior to tolerance induction. Typically, enriched hematopoietic stem cells are stored frozen. A particularly preferred method of cryopreserving said cells is described in co-pending patent application U.S.S.N. 07/780,487, filed October 23, 1991. Briefly, $CD34^+$ cells are suspended in a physiologically acceptable medium containing human plasma, or another source of human or animal protein, and a cryoprotective agent, such as DMSO, and frozen at a controlled rate to a temperature between approximately -80°C and -196°C. Cryopreserved stem cells are preferably thawed immediately prior to transfusion. It is usually desirable to thaw the cells as rapidly as possible, for example, by placing the vessel in which the cells were frozen in a 37°C water bath. Once thawed, the cells may be pelleted by centrifugation, the DMSO-containing freezing medium withdrawn and discarded, and the cells resuspended in a physiologically acceptable medium. The cells may also be washed one or more times, if desired, in a suitable buffer or medium. Since the amount of DMSO used in freezing the cells is small and since some cell loss is inevitable during centrifugation and washing, it is generally preferred to transfuse the cells immediately after thawing, without performing any additional manipulations.

Co-pending U. S. patent application Attorney's Docket No. 15753-2-1, filed April 23, 1993 (and herein incorporated by reference), describes a preferred apparatus for the collection of hematopoietic stem cells directly into a vessel suitable for expansion and/or cryopreservation, which apparatus maintains a closed, sterile field. This apparatus may be advantageously employed in conjunction with the above described methods of expanding and cryopreserving immunoselected stem cells.

Recipient Conditioning

As noted above, within preferred embodiments of the invention enriched stem cells are infused into a recipient which has previously been conditioned for solid-organ transplantation. Briefly, a variety of methods may be utilized in order to condition a recipient for solid-organ transplantation. Representative examples of such methods include immunosuppressive regimens such as administration of anti-lymphocyte serum (ALS), anti-thymocyte globulin (ATG), anti-lymphocyte globulin (ALG), cyclosporine A, FK506, azathioprine, corticosteroids (for example, prednisone), cyclophosphamide, and/or CD3 monoclonal antibody, total lymphoid irradiation, partial lymphoid irradiation, or some combination of the above (for example, ALS, cyclosporin, azathioprine and prednisone are commonly co-administered). Within the context of this disclosure, the terms ALS, ATG, and ALG are used interchangeably.

Within one embodiment of the invention, the recipient is conditioned according to the following regimen: ALS (20 mg/kg body weight intravenously from the first through the seventh to fourteenth day post-transplant) / cyclosporine (3 to 7 mg/kg daily) / prednisone (0.125 mg/kg daily). Typically, ALS is discontinued between days 7 and 14 post-transplant, unless an acute rejection episode occurs, in which case it is resumed until the episode resolves. Prednisone is tapered, typically by 5 mg per week, beginning about week 12 post-transplant. Cyclosporine is generally continued at least until the organ transplant host exhibits mixed chimerism, typically 5 to 8 weeks post-transplant, but may be continued longer. Ideally, the cyclosporine dose is tapered gradually once mixed chimerism has been attained in the host; in some cases, it may even be possible to withdraw the host from cyclosporine altogether.

Infusion of Stem Cells

Following one or more of the procedures discussed above, the enriched stem cells are then infused into a recipient. This may occur either before, coincident with or subsequent to, organ transplantation. For humans, within preferred embodiments of the invention, the stem cell composition is comprised of at least about 30% $CD34^+$ cells, preferably at least about 50% $CD34^+$ cells, and most preferably at least about 60% $CD34^+$ cells. Cell viability of the composition should preferably be at least about 80% and more preferably at least about 90% viable.

The enriched stem cell composition may be administered to the recipient intravenously via a needle and syringe or, via a standard blood administration set. Although a single stem cell may be sufficient for engraftment and may lead to durable chimerism in the host, it is generally preferable to give a larger dose of stem cells in order to maximize the likelihood of engraftment.

Preferably, the enriched cells are provided to the recipient at a concentration of between about 0.3×10^6 and 10×10^6 $CD34^+$ cells/kg, more often between about 1×10^6 and 3×10^6 $CD34^+$ cells/kg, and usually at least about 2×10^6 $CD34^+$ cells/kg. The cells can be administered in any convenient volume, but it is usually preferred to utilize the smallest volume which is practicable. For stem cells selected and enriched in accordance with the methods of this invention, that volume is typically about 5 mls and is usually between about 3 and 10 mls. Since the volume is small compared to the volume of unfractionated marrow (200-1000 mls), infusion into the host can take place relatively rapidly, for example, in a matter of minutes.

Organ Transplantation

A variety of solid-organ grafts may be readily transplanted utilizing the methods and compositions described herein. Representative examples of solid-organ grafts include kidney, lung, liver, heart, pancreas and skin grafts, as well as multi-organ grafts, such as heart and lung, kidney and pancreas, or kidney and liver grafts, and multi-visceral grafts comprised of all intra-abdominal organs. Grafts may be allografts or xenografts. An allograft is defined as a graft from one member of a species (the donor) to another member of the same species (the recipient or host), wherein the donor and recipient may or may not be matched at the major histocompatibility complex (MHC). A xenograft is defined as a graft from a member of one species to a member of a different species. Typically, xenografting is done between phylogenetically related animals, for example, from one mammal, such as a pig or monkey, to another mammal, such as a human. A graft may be primary, meaning that it is the first graft of a given organ type for that recipient, or secondary, meaning that the recipient has received an earlier graft of the same organ type (which was rejected or which ceased to function effectively).

As noted above, organ transplantation may be accomplished either prior to, or subsequent to infusion of the enriched hematopoietic cells. Methods for the preservation and transplantation of organs may vary depending upon the organ to be transplanted and the source of the organ. Such procedures are readily known to those of skill in the art (*see e.g.*, Richard Simmons et al., in *Principles of Surgery*, Schwartz et al., eds., N.Y.: McGraw Hill, pp. 349-442, 1974; Starzle, *Experience in Renal Transplantation*, Philadelphia: L.B. Sanders, 1964; Starzle, *Experience in Hepatic Transplantation*, Philadelphia: L.B. Sanders, 1964; and Najarian and Simmons, *Transplantation*, Philadelphia: Lea and Felsiger, 1972). Within one embodiment of the invention, a kidney may be harvested from a heart-beating donor, and preserved by cold pulsatile perfusion on a Waters MOX 100 machine using a perfusate solution such as Belzer's. The ischemic interval is typically about 18-25 hours.

Assessment of Tolerance Induction

As noted above, the induction of tolerance in a solid organ graft recipient may be assessed in a number of ways, including both *in vitro* and *in vivo* methods. *In vivo*, tolerance can be assessed by long-term acceptance of the grafted organ without concomitant, exogenously supplied immunosuppression.

In vitro, tolerance can be assessed by one-way mixed lymphocyte cultures (MLC) and by cell-mediated lympholysis (CML) activity. A tolerized recipient is expected to exhibit reduced or absent proliferation to donor lymphoid cells in one-way

MLC, while continuing to exhibit proliferation in response to third-party lymphoid cells. Similarly, a tolerized recipient is expected to exhibit absent or reduced cytolysis of donor cells in a CML assay, while maintaining cytolysis of third-party cells.

Methods of performing mixed lymphocyte cultures are well-known in the art and are described, for example, in Mishell and Shiigi (ed.) *Selected Methods in Cellular Immunology*, Philadelphia: WH Freeman, p. 163, 1980. Briefly, blood specimens from putatively tolerized patients are collected in anticoagulant and the mononuclear cells are separated therefrom by means of density gradient centrifugation. The cells are washed in a suitable buffer, such as phosphate buffered saline (PBS), and resuspended to a desired density, typically about 1 X 10^6 cells/ml, in a medium such as RPMI, containing 10% pooled human AB serum and 25 mM HEPES. Cells are plated, at least in triplicate, in 96-well microtiter trays by placing 100 ul in each well. Cells from the putatively tolerized host are referred to as responding cells.

Stimulating cells are mononuclear cells obtained from the spleen of the organ donor and prepared as described above; because the donor is typically cadaveric, the stimulating cells will usually be stored frozen in a suitable medium containing a cryoprotectant and thawed immediately prior to use. If the donor happens to be living, peripheral blood mononuclear cells can be used instead of spleen cells and the requirement for frozen storage may be obviated. Stimulating cells are irradiated with 2500 rads prior to use in MLC; this prevents them from proliferating in culture (hence the term one-way MLC).

Typically, one hundred microliters of stimulating cells are added to each well containing responding cells and the microtiter trays are incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. After approximately 5 days culture, 1 uCi of tritiated thymidine is added to each well containing cells. The cultures are harvested 18-20 hours later and counted in a scintillation counter.

In addition to using donor cells as the stimulating cells, unrelated third party cells and irradiated host cells are also used to control for non-specific proliferation. If tolerance has been established in the host, host cells will not proliferate, as judged by their incorporation of tritiated thymidine, in response to the donor cells to any greater extent than they proliferate in response to autologous or unrelated third party cells.

CML assays are also well-known to those skilled in the art and are described, for example, in Mishell and Shiigi (ibid.), p. 128. Briefly, responding cells and stimulating cells are prepared as described above and co-cultured at 4 X 10^6 cells each in approximately 2 ml of a suitable medium, such as RPMI/10% fetal calf serum (FCS), for 5 days at 37°C. At this time, the cells are harvested, counted, and resuspended at various effector : target ratios with ^{51}Cr-labeled donor cells as targets.

After 3-5 hours, the supernatants are harvested and the specific target cell lysis is quantified relative to appropriate controls. If the host has been tolerized to the donor, there should be no significant chromium release above background.

According to one embodiment of this invention, hematopoietic stem cells are harvested from the bone marrow of an organ donor. The stem cells are partially enriched and reinfused, between days 3 and 5 post-transplant, into an organ graft recipient who has been conditioned for transplantation by one or more methods selected from the group consisting of total lymphoid irradiation (TLI), cyclosporine therapy, CD3 monoclonal antibody therapy, and anti-lymphocyte serum (ALS) therapy, such that a state of mixed chimerism is attained in the organ recipient. Optionally thereafter, the organ recipient may be gradually withdrawn from immunosuppressive therapy.

By utilizing hematopoietic stem cells rather than unfractionated marrow to induce tolerance to donor antigens, several advantages are achieved. First, hematopoietic stem cells are, by definition, capable of self-renewal as well as differentiation into all of the various hematopoietic lineages. Thus, hematopoietic stem cells can mediate a durable state of mixed chimerism. Furthermore, by virtue of their ability to give rise to all of the mature elements of the hematopoietic system, stem cells can mediate tolerance by any of a variety of mechanisms, including active T cell-mediated suppression, clonal deletion, and clonal anergy. This is important since the precise mechanism by which tolerance is induced and maintained is not known; different mechanisms may be important at different times (for example, immediately post-transplant and much later) and may be mediated by more than one cell type.

Second, by transfusing only hematopoietic stem cells, which are functionally immature cells, the possibility of sensitizing the host and precipitating acute graft rejection is greatly diminished, these phenomena being mediated by mature cells. Likewise, the likelihood of eliciting GVHD, which is mediated predominantly by mature T cells, is markedly less when stem cells rather than whole marrow are used to tolerize the host.

In a preferred embodiment of the instant invention, hematopoietic stem cells are harvested from the bone marrow of an organ donor and enriched by means of immunoselection for $CD34^+$ cells. The resultant cells are cryopreserved by freezing at a controlled rate to a temperature between approximately -80 and -196°C in a freezing medium comprising a physiologically acceptable medium in combination with a penetrating cryoprotectant and a source of protein. Between five and seven days post-organ transplantation, the cryopreserved, enriched $CD34^+$ cells are thawed and reinfused into an organ recipient who has been conditioned since transplant by administration of ALS and cyclosporine. At approximately fourteen days post-organ

transplantation, ALS therapy is discontinued. Cyclosporine administration is continued at least until such time as the recipient attains a state of mixed chimerism, after which the cyclosporine dose may be tapered gradually and possibly even withdrawn.

Assessment of Mixed Chimerism

As noted above, the present invention provides methods and compositions suitable for tolerizing a recipient for solid-organ transplantation. In most (but not all cases), this produces a state of mixed chimerism (a state in which there is a stable mixture of host and donor hematopoietic elements). Briefly, there are a number of methods by which one skilled in the art can determine whether a solid organ graft recipient has attained a state of mixed chimerism. Typically, this is accomplished by isolating hematopoietic cells from the peripheral circulation and subjecting them to analysis by immunophenotyping. Other methods include mini-satellite detection, as described in Harano et al. (*Bone Marrow Transplant 12*:221, 1993), karyotyping and determination of immunoglobulin allotypes.

Within one embodiment of the invention, a blood specimen is obtained from a putatively tolerized recipient by phlebotomy. Usually, the blood is collected in an anticoagulant, such as EDTA, heparin, ACD, or CPDA. The blood specimen is centrifuged and the buffy coat which forms at the interface of the red cell and plasma layers is harvested. The buffy coat may be fractionated by density gradient centrifugation, for example, using Ficoll-Hypaque or Percoll, to yield a mononuclear cell (MNC) fraction. The MNC fraction is washed in a suitable buffer or medium and the cells are resuspended to a desired density. Aliquots of cells are stained with various directly or indirectly labeled antibodies capable of distinguishing between cells of donor origin and cells of recipient origin (such as antibodies to private MHC determinants), and the resultant stained cell suspension is interrogated, for example, using a fluorescence activated cell sorter (FACS) or other means. If the solid organ graft recipient is a mixed chimera, hematopoietic cells expressing donor antigens, as well as hematopoietic cells expressing host antigens should be detected.

The proportion of chimerism may vary from less than 1% donor cells to approximately 90% donor cells. The relative proportions of donor and host cells may vary as a function of the time post-transfusion. Typically, chimerism will be evident in the host within approximately 15-45 days post-transfusion, although the time course may vary widely among individuals. In some instances, it may be difficult to demonstrate chimerism in the peripheral circulation, but be possible to demonstrate it in other blood-forming organs, such as the spleen or the marrow. Accordingly, if chimerism cannot be demonstrated in one tissue at one time point, it may be desirable to

re-assay the same tissue at a later time point(s) or to assay a different tissue(s) before drawing a conclusion. In general, it is believed that a level of chimerism as low as 1% in any recipient tissue may be sufficient to induce tolerance in the host to donor-derived antigens.

Utilizing the methods and compositions of the instant invention, it is possible to tolerize a solid organ graft recipient to donor antigens, such as are expressed by the graft, thereby reducing or eliminating the need for expensive and, in many cases, toxic immunosuppressive agents in the host. Examples of toxicities associated with commonly used immunosuppressive agents include liver toxicity (cyclosporine, azathioprine), renal toxicity (cyclosporine), leukopenia and thrombocytopenia (total lymphoid irradiation, azathioprine, cyclophosphamide, actinomycin), hypertension (prednisone), and growth retardation (prednisone, actinomycin, total lymphoid irradiation). In addition, chronic immunosuppression, such as is typically required to maintain graft acceptance, can lead to an increased incidence of infections, including viral (EBV, CMV, VZV, etc.), bacterial, and fungal (Aspergillus, Nocardia, Toxoplasma, Cryptococcus, etc.) infections, and malignancy in the host.

Further, the methods and compositions of the instant invention enable organ transplantation between unrelated individuals, without the need for matching donor and recipient. Thus, the instant invention provides multiple substantial advantages over other art-accepted methods of maintaining solid organ graft survival and function.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A method for tolerizing a recipient for solid-organ transplantation, comprising:

(a) harvesting cells from an anticipated donor of a solid-organ, said cells including hematopoietic stem cells;

(b) enriching hematopoietic stem cells from said harvested cells; and

(c) infusing said enriched stem cells into a recipient, such that a state of tolerance to a transplanted solid-organ from said donor is induced in said recipient.

2. A method for tolerizing a recipient for solid-organ transplantation, comprising:

(a) harvesting cells from an anticipated donor of a solid-organ, said cells including hematopoietic stem cells;

(b) enriching hematopoietic stem cells from said harvested cells;

(c) conditioning a recipient for solid-organ transplantation; and

(d) infusing said enriched stem cells into said conditioned recipient, such that a state of tolerance to a transplanted solid-organ from said donor is induced in said recipient.

3. The method of claims 1 or 2 further comprising, subsequent to the step of enriching said stem cells and prior to the step of infusing said enriched stem cells into a recipient:

(a) freezing said enriched stem cells; and

(b) thawing said frozen stem cells.

4. The method of claim 2 wherein said recipient is conditioned by administration of an immunosuppressive agent selected from the group consisting of anti-lymphocyte serum, anti-thymocyte globulin, anti-lymphocyte globulin, cyclosporine A, FK506, azathioprine, and cyclophosphamide.

5. The method of claim 2 wherein said recipient is conditioned by administration of a corticosteroid.

6. The method of claim 2 wherein said recipient is conditioned by irradiation.

7. The method of claim 2 wherein said recipient is conditioned by CD3 monoclonal antibody therapy.

8. The method of claims 1 or 2 wherein said solid-organ is selected from the group consisting of kidney, lung, liver, heart, pancreas and skin.

9. The method of claims 1 or 2 wherein said stem cells are enriched from said harvested cells by immunoselection.

10. The method of claims 1 or 2 wherein said stem cells are $CD34^+$ cells.

11. The method of claims 1 or 2 further comprising, subsequent to the step of enriching said stem cells, expanding the number of stem cells *in vitro*.

12. The method of claims 1 or 2 wherein said harvested cells are selected from the group consisting of bone marrow, mobilized peripheral blood, fetal liver, and cord blood.

13. A method for transplanting solid organs, comprising:

(a) harvesting cells from an anticipated donor of a solid-organ, said cells including hematopoietic stem cells;

(b) enriching hematopoietic stem cells from said harvested cells;

(c) infusing said enriched stem cells into an anticipated solid-organ graft recipient, such that a state of tolerance to a solid-organ from said donor is induced in said recipient; and

(d) transplanting an organ from said donor into said recipient.

14. A method for transplanting solid organs, comprising:

(a) harvesting cells from an anticipated donor of a solid-organ, said cells including hematopoietic stem cells;

(b) enriching hematopoietic stem cells from said harvested cells;

(c) transplanting an organ from said donor into a recipient; and

(d) infusing said enriched stem cells into said recipient, such that a state of tolerance to a solid-organ from said donor is induced in said recipient.

15. A method for transplanting solid organs, comprising:

(a) harvesting cells from an anticipated donor of a solid-organ, said cells including hematopoietic stem cells;

(b) enriching hematopoietic stem cells from said harvested cells;

(c) conditioning a recipient for solid-organ transplantation;

(d) infusing said enriched stem cells into said conditioned recipient, such that a state of tolerance to a solid-organ from said donor is induced in said recipient; and

(e) transplanting an organ from said donor into said recipient.

16. A method for transplanting solid organs, comprising:

(a) harvesting cells from an anticipated donor of a solid-organ, said cells including hematopoietic stem cells;

(b) enriching hematopoietic stem cells from said harvested cells;

(c) conditioning a recipient for solid-organ transplantation;

(d) transplanting an organ from said donor into said conditioned recipient; and

(e) infusing said enriched stem cells into said conditioned recipient, such that a state of tolerance to a solid-organ from said donor is induced in said recipient.

17. The method of claims 13 - 16 further comprising, subsequent to the step of enriching said stem cells and prior to the step of infusing said enriched stem cells into a recipient:

(a) freezing said enriched stem cells; and

(b) thawing said frozen stem cells.

18. The method of claims 13 - 16 wherein said recipient is conditioned by administration of an immunosuppressive agent selected from the group consisting of anti-lymphocyte serum, anti-thymocyte globulin, anti-lymphocyte globulin, cyclosporine A, FK506, azathioprine, and cyclophosphamide.

19. The method of claims 13 - 16 wherein said recipient is conditioned by administration of a corticosteroid.

20. The method of claims 13 - 16 wherein said recipient is conditioned by irradiation.

21. The method of claims 13 - 16 wherein said recipient is conditioned by CD3 monoclonal antibody therapy.

22. The method of claims 13 - 16 wherein said solid-organ is selected from the group consisting of kidney, lung, liver, heart, pancreas and skin.

23. The method of claims 13 - 16 wherein said stem cells are enriched from said harvested cells by immunoselection.

24. The method of claims 13 - 16 wherein said stem cells are $CD34^+$ cells.

25. The method of claims 13 - 16 further comprising, subsequent to the step of enriching said stem cells, expanding the number of stem cells *in vitro*.

26. The method of claims 13 - 16 wherein said harvested cells are selected from the group consisting of bone marrow, mobilized peripheral blood, fetal liver, and cord blood.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 09234 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 13 May 1993 ---	
A	WO,A,92 18615 (INDIANA UNIVERSITY FOUNDATION) 29 October 1992 -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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